Plant Physiol. (1988) 88, 993–995
0032-0889/88/88/0993/03/$01.00/0

Communication

Evidence for the Nuclear Location of the Genes for Chloroplast IF-2 and IF-3 in *Euglena*[1]

Received for publication May 27, 1988 and in revised form August 5, 1988

BETSY L. KRAUS AND LINDA L. SPREMULLI*
Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina 27599

ABSTRACT

The chloroplast translational initiation factors IF-2 and IF-3 from *Euglena gracilis* are present in low levels in dark-grown cells and can be induced by exposure of cells to light. Studies of the antibiotic sensitivity of the light induction of these factors indicates that both are encoded in the nuclear genome.

Chloroplasts contain a translational system which synthesizes proteins encoded in the chloroplast genome. However, only a small percentage of the proteins found within the chloroplast are actually encoded in the organelle DNA. The genetic information for most chloroplast proteins is located in the nucleus of the cell. These proteins are synthesized in the cytoplasm and subsequently transported into the chloroplast. The proteins of the chloroplast translational system follow this same basic trend. About 70% of chloroplast ribosomal proteins are encoded in the nucleus while the remainder are encoded within the organellar DNA (7, 8). Two of the chloroplast elongation factors, EF-G and EF-Ts, are nuclearly encoded (1, 2) while the gene for EF-Tu is located in the chloroplast genome in *Chlamydomonas reinhardii* and *Euglena gracilis* (11, 14) and in the nuclear genome in tobacco and liverwort (6, 9).

We are interested in the location of the genetic information for the chloroplast protein synthesis initiation factors. Two *E. gracilis* chloroplast initiation factors, IF-2_{chl} and IF-3_{chl}, have recently been characterized (3, 5). Chloroplast IF-2 is responsible for the binding of the initiator tRNA to the ribosome. It appears to be present in two different forms in *Euglena*. IF-2_{chl} functions with its homologous chloroplast ribosomes but not with *Escherichia coli* ribosomes. Chloroplast IF-3 promotes ribosome dissociation and functions on either chloroplast or *E. coli* ribosomes. Initiation factor 1 which is expected to enhance the activities of IF-2_{chl} and IF-3_{chl} has not yet been detected in chloroplasts. However, a gene for a putative IF-1_{chl} has been localized to the chloroplast genomes of liverwort, tobacco, and spinach (6, 9, 10) by homology to *E. coli* IF-1.

In order to obtain information on the gene location of IF-2_{chl} and IF-3_{chl} in *Euglena*, we have examined the light induction of these two factors in the presence of antibiotics which inhibit protein synthesis. When *Euglena* is grown in the dark, the chloroplast does not develop into the mature organelle but remains as a proplastid structure. Under these conditions, many of the components of the chloroplast including the translational machinery are greatly reduced. However, upon exposure of the cells to light, chloroplast development is induced and the mature chloroplast develops over about a 72 h period (13). The development of the mature chloroplast is accompanied by the induction of the protein synthetic machinery of this organelle. The differential antibiotic sensitivity of organelle and cytoplasmic protein synthesis can, thus, be used to gain some insight into the site of translation and, thus the location of the genetic information, for these two initiation factors.

EXPERIMENTAL PROCEDURES

Growth of *E. gracilis*. Stock cultures of *Euglena gracilis klebs* var *barcillaris Cori* (*Euglena* B) were maintained in the dark in Hutner's pH 3.5 heterotrophic medium (4). Ten mL aliquots of the stock cultures were inoculated into 1 L of Hutner's media and grown in the dark with agitation to a cell density of 1.5 to 3.0×10^6 cells/mL. The cultures were transferred to 2.5 L of resting media (12) adjusted to pH 5.0 or 7.0 with KH_2PO_4 and K_2HPO_4 and grown in the dark with shaking for an additional day. The cells were then illuminated for 3 d with four cool-white (GE) and four Grolux (Sylvania) fluorescent bulbs. Where indicated, antibiotics were added 1 to 6 h prior to exposure to light. Control cultures were maintained in the dark for an equivalent period of time and are termed dark-grown cultures. Cells were harvested by centrifugation, washed in buffer A (50 mM Tris-HCl [pH 7.8], 50 mM NH_4Cl, and 5 mM $MgCl_2$), fast frozen in a dry ice/2-propanol bath and stored as cell pellets at −70°C until use.

Preparation of Cell Extracts and Determination of IF-3_{chl} Activity. Extracts containing IF-3_{chl} were prepared as described previously (5) except that the initial buffer used contained 50 mM Tris-Cl [pH 8.5], 50 mM NH_4Cl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 μM GDP, 10% glycerol, and 12 mM 2-mercaptoethanol and the extracts were dialyzed against buffer B (10 mM Tris-Cl [pH 8.5], 10 mM NH_4Cl, 0.1 mM EDTA, 10% glycerol, and 12 mM 2-mercaptoethanol). Prior to the determination of IF-3_{chl} activity, extracts were partially purified by chromatography on DEAE-cellulose. In this procedure, a 3 mL DEAE-cellulose column (0.86 × 5.2 cm) in a 3 mL syringe was equilibrated with buffer B. The dialyzed extract (10–18 mL, 90–150 mg) was applied at a flow rate of 2.5 mL/min. The column was washed with 15 mL buffer B and then eluted with a 15 mL linear gradient (10–500 mM NH_4Cl in buffer B) at a flow rate of 2.5 mL/min. Fractions (0.8 mL each) were collected in siliconized tubes and assayed for IF-3_{chl} (5) and EF-Tu_{chl} (11) activity.

Preparation of Extracts and Column Chromatography for the Determination of IF-2_{Chl} Activity. Cells were grown as described above except that they were washed in buffer containing 50 mM Tris-HCl [pH 8.5], 50 mM NH_4Cl, and 25 mM $MgCl_2$. Extracts

[1] Supported in part by funds provided by the National Institutes of Health grant GM 24963.

were prepared as described (3) and the postribosomal supernatants were dialyzed against buffer C (50 mM Tris-HCl [pH 7.8], 50 mM NH_4Cl, 0.1 mM EDTA, 6 mM β-mercaptoethanol, and 10% glycerol). For the determination of IF-2_{chl} activity approximately 44 mg of the postribosomal supernatant from each extract was applied to a 2 cc phosphocellulose column (0.86 × 3.4 cm) equilibrated in buffer C. The column was developed with this buffer until the absorbance at 280 nm returned to baseline. A sample of buffer C containing 0.4 M NH_4Cl was then applied to the column at a flow rate of 0.4 mL/min. Fractions of 0.9 mL were collected in siliconized tubes and appropriate fractions were tested for IF-2_{chl} activity (3). Fractions were pooled, fast frozen, and stored at −70°C. IF-$2_{chl\alpha}$ and IF-$2_{chl\beta}$ were separated by chromatography on DEAE-cellulose. For this procedure, the pooled fractions from the phosphocellulose column containing IF-2 activity were dialyzed against two changes of 125 mL each of buffer C. The samples were applied to a 0.5 mL column of DEAE cellulose equilibrated in buffer C prepared in a Pasteur pipette. The column was developed with buffer C until all the nonretained protein (including IF-$2_{chl\alpha}$) had been eluted and was then developed with buffer C containing 0.4 M NH_4Cl to elute IF-$2_{chl\beta}$. Fractions (0.5 mL) were collected at a flow rate of 0.4 mL/min into 1.5 mL Eppendorf tubes and aliquots of appropriate fractions tested for IF-2_{chl} activity.

RESULTS AND DISCUSSION

Location of the Genetic Information for Chloroplast IF-3. When *Euglena* is exposed to light, plastid development is induced along with the components of the chloroplast translational system. Antibiotics that inhibit chloroplast protein synthesis will prevent the light induction of proteins encoded within the chloroplast genome and can be used to investigate the site of synthesis of proteins localized within this organelle. The determination of the site of synthesis of the chloroplast initiation factors has been complicated by the difficulty of isolating intact chloroplasts from this organism and by the low levels of these factors in whole cell extracts. For technical reasons we have developed separate procedures for the determination of the amounts of IF-2_{chl} and IF-3_{chl} in extracts prepared from cells light-induced for chloroplast development in the presence and absence of antibiotics. Samples enriched for IF-3_{chl} activity were prepared by DEAE-cellulose chromatography of the postribosomal supernatant. The activity of IF-3_{chl} present in cells maintained in the dark or in which chloroplast development had been induced by exposure to light is summarized in Table I. IF-3_{chl} activity was not detectable in the extracts from dark grown cells. In contrast, substantial activity was observed in extracts from light induced cells. When cells were light-induced in the presence of chloramphenicol, an inhibitor of chloroplast protein synthesis, the IF-3_{chl} activity detected was comparable to that observed in the absence of antibiotics. Similar results were obtained when streptomycin, another inhibitor of chloroplast protein synthesis, was tested (data not shown). These results suggest that protein synthesis within the chloroplast is not required for the light induction of IF-3_{chl}. Since the expression of chloroplast genes requires chloroplast protein synthesis, these results indicate that IF-3_{chl} is synthesized in the cytoplasm and, hence, is the product of a nuclear gene. Cycloheximide prevents the induction of chloroplast IF-3. This result is expected since cycloheximide inhibits the expression of nuclear encoded genes which are required for chloroplast development.

Table I. *Effect of Light and Antibiotics on the Induction of IF-3_{chl}*

Cultures were grown, and IF-3 and EF-Tu levels were determined as described in "Materials and Methods." The Chl content of whole cells and EF-G levels in the postribosomal supernatant were measured as described previously (1). The data were normalized to units detected per cell.

Growth Conditions	IF-3	EF-Tu	EF-G	Chl
	units/cell	*units/cell*	*units/cell*	*pg/cell*
Dark grown[a]	$<2 \times 10^{-9}$	60×10^{-9}	1.4×10^{-5}	<0.1
Light induced[b]	27×10^{-9}	400×10^{-9}	4.7×10^{-5}	9.3
+ Chloroamphenical[a]	31×10^{-9}	66×10^{-9}	5.1×10^{-5}	1.4
+ Cycloheximide[a]	$<2 \times 10^{-9}$	56×10^{-9}	1.5×10^{-5}	<0.1

[a] The average of results from the extracts of two 3.5 L cultures, one grown in pH 5.0 resting media, the other in pH 7.0 resting media. [b] The average of results from the extracts of three 3.5 L cultures, one grown in pH 5.0 resting media and two grown in pH 7.0 resting media.

Table II. *Effect of Light and Antibiotics on the Induction of IF-2_{chl}*

Induction Conditions	Inhibitor	Chl/Cell	IF-2_{chl}[a]
		pg/cell	*units*
Light		6.9	28
Dark		<0.2	6.8
Light	Chloramphenicol	0.4	17
Light	Cycloheximide	<0.1	7

[a] Measured after chromatography on phosphocellulose as described in "Materials and Methods." The values obtained have been normalized for identical mg of postribosomal supernatant applied to the column.

As a control, we have compared the results obtained with IF-3_{chl} to the data obtained for *E. gracilis* chloroplast EF-Tu (a chloroplast encoded protein) and chloroplast EF-G (a nuclear encoded protein). As indicated in Table I, the light induction of EF-Tu_{chl} is inhibited by both chloramphenicol and cycloheximide as expected for a chloroplast encoded protein. In contrast, the light induction of EF-G_{chl} is not inhibited during chloroplast development in the presence of chloramphenicol (Table I) but is inhibited by the presence of cycloheximide. Chloroplast IF-3 and EF-G activities clearly follow the same pattern, indicating that IF-3_{chl} is encoded in the nuclear genome.

Determination of the Location of the Genetic Information for IF-2_{chl}. The low levels of IF-2_{chl} activity in the postribosomal supernatant prevented a direct determination of the effects of various antibiotics on the light induction of this factor. Furthermore, the direct DEAE-cellulose chromatographic procedure used for the determination of IF-3_{chl} activity could not be used for the determination of IF-2_{chl} activity because of the presence of a potent inhibitor of initiation complex formation cochromatographing with IF-2_{chl} on this resin. Hence, chromatography on phosphocellulose was used to partially purify this chloroplast initiation factor from other proteins. IF-2_{chl} is retained by phosphocellulose and can be separated from a number of contaminating proteins by this procedure (3). Phosphocellulose column preparations from light-induced cells show a substantial amount of IF-2_{chl} activity (Table II). In contrast, extracts from cells grown and maintained in the dark show about fivefold lower levels of this factor. The IF-2_{chl} activity detected in extracts of dark grown cells may represent levels of this factor present in proplastids or may be the result of other, chloroplast independent activities in the cell that give a positive response in the assay used. When cells are exposed to light in the presence of cycloheximide, Chl synthesis is abolished and there is no apparent induction of IF-2_{chl} activity (Table II). The levels detected in cycloheximide treated cells are essentially identical to those observed in cells maintained in the dark (data not shown). However, when cells are exposed to light in the presence of chloramphenicol, an inhibitor of the chloroplast protein synthesizing system, phosphocellulose preparations contain a substantial amount of IF-2_{chl} activity (Table II). The amount of IF-2_{chl} activity observed

in these preparations is significantly above that observed in extracts from dark-grown cells but is only about 60% of that observed in cells exposed to light in the absence of antibiotics. The presence of substantial amounts of IF-2_{chl} activity in extracts from chloramphenicol treated cells indicates that a gene from this factor must reside with the nuclear genome.

We have recently observed two forms of IF-2_{chl} (designated IF-$2_{chl\alpha}$ and IF-$2_{chl\beta}$) which can be separated by chromatography on DEAE-cellulose (our unpublished observations). The relationship between these forms is not clear at the present time. The lower level of IF-2_{chl} in chloramphenicol treated cells could indicate the presence of two genes for IF-2_{chl}, one of which resides within the nuclear genome and the other of which resides within the chloroplast DNA. In order to examine this possibility and to determine whether the genetic information for both IF-$2_{chl\alpha}$ and IF-$2_{chl\beta}$ was located within the nuclear genome, phosphocellulose preparations of IF-2_{chl} were subjected to chromatography on DEAE-cellulose and the amounts of IF-$2_{chl\alpha}$ and IF-$2_{chl\beta}$ were determined. Both forms of this factor could be detected in extracts of cells exposed to the light in the presence of chloramphenicol (data not shown). The amounts of both forms are reduced in the presence of chloramphenicol. The presence both IF-$2_{chl\alpha}$ and IF-$2_{chl\beta}$ in the cell treated with this antibiotic suggests that both forms of IF-2_{chl} are products of nuclear gene(s). The partial reduction of IF-2_{chl} in extracts of chloramphenicol treated cells could arise from the partial degradation of this factor when it cannot be localized in the mature chloroplast following its synthesis in the cytoplasm.

The results presented here demonstrate that both IF-2_{chl} and IF-3_{chl} are synthesized outside of the chloroplast on cytoplasmic ribosomes indicating that both initiation factors are encoded in the nuclear genome in *Euglena*. No sequences homologous to prokaryotic IF-2 and IF-3 were observed in the chloroplast genomes of tobacco and liverwort suggesting that the genes for these two proteins are also located in the nucleus in higher plants (6, 9). However, homology searches may have failed to reveal the presence of these genes in the chloroplast genome. Lack of strong homology may be an especially important problem with IF-3_{chl} which has very different physical properties from those of *E. coli* IF-3 (5). Interestingly, sequences homologous to *E. coli* IF-1 have been identified in the chloroplast genomes of spinach, tobacco, and liverwort (6, 9, 10). The dispersed pattern of gene location observed with the initiation factors conforms with that observed for chloroplast proteins in general.

LITERATURE CITED

1. Breitenberger CA, MC Graves, LL Spremulli 1979 Evidence for the nuclear location of the gene for chloroplast elongation factor G. Arch Biochem Biophys 194: 265–270
2. Fox L, J Erion, J Tarnowski, L Spremulli, N Brot, H Weissbach 1980 *Euglena gracilis* chloroplast EF-Ts: evidence that it is a nuclear encoded gene product. J Biol Chem 255: 6018–6019
3. Gold JG, LL Spremulli 1985 *Euglena gracilis* chloroplast initiation factor 2: identification and initial characterization. J Biol Chem 260: 14897–14900
4. Greenblatt C, J Schiff 1959 A pheophytin-like pigment in dark-adapted *Euglena gracilis*. J Protozool 6: 23–28
5. Kraus BL, LL Spremulli 1986 Chloroplast initiation factor 3 from *Euglena gracilis*: identification and initial characterization. J Biol Chem 261: 4781–4784
6. Ohyama K, H Fukuzawa, T Kohchi, H Shirai, T Sano, S Sano, K Umesono, Y Shiki, M Takeuchi, Z Chang, S Aota, H Inokuchi, H Ozeki 1986 Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. Nature 322: 572–574
7. Posno M, M van Noort, R Debise, G Groot 1984 Isolation, characterization, phosphorylation and site of synthesis of *Spinacia* chloroplast ribosomal proteins. Curr Genet 8: 147–154
8. Schmidt R, C Richardson, N Gillham, J Boynton 1983 Sites of synthesis of chloroplast ribosomal proteins in *Chlamydomonas*. J Cell Biol 96: 1451–1463
9. Shinozaki K, M Ohme, M Tanaka, T Wakasugi, N Hayashida, T Matsubayashi, N Zaita, J Chunwongse, J Obokata, K Yamaguchi-Shinozaki, C Ohto, K Torazawa, B Meng, M Sugita, H Dena, T Kamogashira, K Yamada, J Kusuda, F Takaiwa, A Kata, N Tohdoh, H Shimada, M Sugiura 1986 The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. EMBO 5: 2043–2049
10. Sijben-Muller G, RB Hallick, J Alt, P Westhall, RG Herrmann 1986 Spinach plastid genes coding for initiation factor IF-1, ribosomal protein S1 and RNA polymerase α-subunit. Nucleic Acids Res 14: 1028–1044
11. Spremulli LL 1982 Chloroplast elongation factor Tu: evidence that it is the product of a chloroplast gene in *Euglena*. Arch Biochem Biophys 214: 734–741
12. Sreedharan S, CM Beck, LL Spremulli 1985 *Euglena gracilis* chloroplast elongation factor Tu: purification and initial characterization. J Biol Chem 260: 3126–3131
13. Stern A, J Schiff, H Epstein 1964 Studies of chloroplast development in *Euglena*. V. Pigment biosynthesis, photosynthetic oxygen evolution and carbon dioxide fixation during chloroplast development. Plant Physiol 39: 220–226
14. Watson JC, S Surzycki 1982 Extensive sequence homology in the DNA coding for elongation factor Tu from *Escherichia coli* and the *Chlamydomonas reinhardtii* chloroplast. Proc Natl Acad Sci USA 79: 2264–2267